THE UNIVERSITY OF ALBERTA

STUDIES ON THE DETECTION OF ANTIBIOTICS IN RAW AND PROCESSED MILK

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF DAIRY SCIENCE

by
H.M. MEI, B.Sc.
EDMONTON, ALBERTA
AUGUST, 1963.

ABSTRACT

A comparison has been made of the tests for antibiotics in milk to judge which method might be most suitable for modification and various methods have been tried to arrive at a more sensitive test for penicillin in milk. The use of cobalt and surface active agents on the test organisms Streptococcus thermophilus and Bacillus subtilis in conjunction respectively with the dye reduction and disc assay methods, had no apparent effect in increasing the sensitivity or speeding up the tests. Irradiation of the above two microorganisms had no detectable effect in rendering them more susceptible to the action of penicillin.

Improvements have been achieved in increasing the sensitivity of the test by different methods of concentrating the penicillin in milk to be tested. Earlier workers (Kosikowski & Mocquot, 1956) had shown that the sensitivity could be increased ten-fold by a vacuum drying technique whereby the milk powder was formed into tablets which were used in the disc assay instead of filter paper discs impregnated with the test sample. However the vacuum freeze-drying technique requires several hours to prepare the sample. A speedier method of concentrating the antibiotic has been worked out by separation of the whey from a test milk sample

by rennet coagulation and concentration of the whey by a rotary film evaporator at 55° under vacuum. A 10:1 reduction in volume could be obtained in about 7 min. Milk containing 0.003 iu/ml of penicillin could be detected by using the existing modified disc assay method. By doubling the volume of the sample 0.0015 iu/ml could be detected.

The whey concentration technique has been satisfactorily used for detecting minimal quantities of penicillin in market milk and milk products. In a survey of market milk, condensed milk and dried milk, several samples were found to contain penicillin. Accordingly it is suggested that more stringent control should be exercised in dairy plants for the detection of antibiotics in raw milk.

ACKNOWLEDGEMENTS

This study was made possible by the financial support of a National Research Council Assistantship. For this, I wish to express my sincere thanks to the authorities concerned.

I am deeply indebted to Dr. L.F.L. Clegg for his help and encouragement throughout my study.

TABLE OF CONTENTS

<u>LIST OF TABLES</u>

LIST OF PLATES

REVIEW OF LITERATURE

Incidence of Antibiotics in Market Milk Supplies

Antibiotics have been used by dairymen and veterinarians for over a decade in the treatment of diseases of dairy cattle. They are administered to dairy cattle via several routes: (1) infusion into the udder through the streak canal for the treatment of mastitis, (2) intramuscular and intravenous injection for the treatment of numerous diseases, and (3) orally, for treatment or prevention of diseases or as a dietary supplement. Antibiotics in milk are largely the result of failure to discard milk from treated quarters or failure use it for purposes other than human consumption until 72 hr after the last treatment. (Grove, 1959; Welch, 1957).

Several surveys of antibiotics in milk have been carried out in the United States, Britain and Canada. Albright *et al.*(1961) have summarized some national and local surveys on the presence of antibiotics in the milk supply before and after 1960 in the United States. During the years 1951-1959, twelve surveys were made and 377 positive samples were found out of 7,201 samples tested (5.2%). The concentration of penicillin found in milk ranged from 0.003-0.55 international units (iu)/ml. In 1960, two surveys revealed that

4,133 positive samples were found in 768,468 samples tested (0.54%).

In Britain surveys covering a five year period 1951-1955 have been made (Berridge, 1956; Storrs & Hiett-Brown, 1954). The data indicated that a range 1.4-17.5% of samples examined contained antibiotics. Surveys made in Canada in 1952 (Johns, 1953), indicated 1.3-1.5% of samples tested contained antibiotics. The data reported by Dairy Branch, Department of Agriculture, Government of the Province Alberta are shown in Table 1.

Table 1. Incidence of antibiotics in Alberta milk - August 1st to November 30th, 1960. (Government of Alberta, 1960)

Location	No. of herds	No. of negative samples	No. of positive samples	Incidence of antibiotics (%)
Edmonton	434	430	4	
Calgary	364	361	3	
Lethbridge	54	54	0	
Country Plants	165	164	1	
Total	1,017	1,009	8	0.78

Problems Created by Antibiotics in Milk Supplies

The presence of antibiotics in milk has created important problems in the dairy industry and to public health officials (e.g. Marth & Ellickson, 1959). The problems affecting the dairy industry are: (1) failure of starter culture growth in reconstituted skim milk for cheese and cultured milks, (2) slowness or failure of "setting" of the milk in cheese manufacture and subsequent ripening, (3) in acid and flavor production during the manufacture of butter-milk and other cultured milks, and (4) the validity of some bacteriological quality control tests.

Public health hazards associated with consumption of milk and milk products contaminated with antibiotics include: (1) allergic response, (2) change in intestinal flora, and (3) development of antibiotic-resistant pathogenic bacteria.

From the point of view of the dairy industry, very small quantities of antibiotics in milk may be harmful to the product and to the public health. The Food and Drug Administration in the U.S.A. requires a complete absence of added antibiotic in milk. The definitions of "small quantities" and "complete absence" respectively however depends on the sensitivity of the method used for the detection of antibiotics (Storgards & Mohr, 1962).

Mode of Action of Antibiotics

In order to assess the suitability of various tests for antibiotics in milk, it is desirable to review existing knowledge of the action of antibiotics on micro-organism.

Eagle & Saz (1955) have indicated that the mechanisms of the cytotoxic action of antibiotics remains obscure. Goldberg (1959) suggested that in the present state of our knowledge, the mode of action of antibiotics could not be stated except as a series of part truths. He suggested that the action of all antibiotic was the same. Antibiotics were either adsorbed onto or absorbed into the cell, and they inhibited the effective growth of the culture in relatively low concentration. They exhibited some selectivity or differential toxicity under different conditions of use to a variety of bacterial cells.

It was postulated that antibiotics worked directly or indirectly on the bacteria in many possible ways: (1) changing the permeability of cell wall or causing lysis; (2) affecting the metabolism by interrupting one or more enzymatic processes; (3) interfering with the reproductive processes; (4) affecting excretion processes; (5) altering the resistance to toxic chemicals; (6) by weakening the defense systems; and (7) by interfering with control of homeostasis in any of the other processes considered.

Goldberg (1959) has classified antibiotics as surfactants and antimetabolites. Presumably antibiotics which have surface tension reducing properties adhere selectively to cell walls, membranes or interfaces, and reduce the cohesive force between the molecules which form that surface. The resultant reduction in surface energy increases cell permeability and increases the tendency of the membrane-forming molecules to dispere into the surrounding medium, to the extent that the integrity of the membrane may be lost; and for lack of a membrane the cell is destroyed.

Antibiotics are said to act as antimetabolites usually by combining with an enzyme. Although there is certainly too little data of a quantitative nature to support the views that many antibiotics act as specific enzyme inhibitors, some have such properties, and others are suspected of being metabolic antagonists.

Classic examples of competitive inhibition are the inhibition of the utilization of p-amino benzoic acid by sulfanilamide, or the blockage of the succinic acid dehydrogenase reaction by malonic acid. As the formulae of these compounds indicate, the space occupied, general configuration, overall composition, and placement of similar reactive groups of the inhibitor are similar to those of the actual metabolite; therefore it is easy to see how the enzyme reacts with one as well as the other.

The success of the sulfa-drugs as competitive antimetabolites has greatly influenced thinking and research in antibiotics. Although several antibiotics are found to be competitive inhibitors of certain metabolites, it has not been shown that this is their mode of action as a selective inhibitor.

Antibiotics may disrupt and decrease sulfhydryl groups of the bacterial cell. Many enzymes depend on the integrity of the sulfhydryl group for intramolecular function or for interaction with other molecules. These include urease, papain, myosin, β-amylase and succinoxidase (Neilands & Stumpf, 1955).

Cooper (1956) has summarized the probable sequence of the effects of penicillin on bacterial growth as a timetable starting with the addition of penicillin to a growing culture.

Time, 0-2 min: All penicillin binding components (PBC) are fixed and bacterial reproduction is stopped at the lowest concentration (0.1 iu/ml).

Time, 2-30 min: No death or lag in resumption of growth occurs if penicillin is removed at this time. The amount of penicillin bound is doubled with a concomitant loss of internal PBC. The rate of resumed growth is slower.

Time, 30-60 min: The viable counts begin to decrease in an exponential manner. The bound penicillin/unit volume

of culture still increases, but the rate of binding decreases steadily. The cell ceases to accumulate gross dry weight, and such constituents as Na^+, Mg^{++}, K^+, H_2PO_4 and glutamate. The lag in growth resumption increases with the time spent in contact with penicillin. Removal of penicillin prevents further cells dying. Cell wall material and cell protein are no longer synthesized but synthesis of nucleic acid and peptide continues. Uptake of Co^{++}, Fe^{++} and total phospholipids synthesis are unaffected.

Time, 60-75 min: Nucleic acid and peptide synthesis slows down.

Time, 75 min: The cell swells and loses solutes, and the inclusion of phosphate into large molecules decreases. The dissolution of the cell commences.

Methods of Detecting Antibiotics in Milk

Chemical methods

The sensitivity of the chemical methods developed for the determination of antibiotics is generally low compared with that of microbiological methods. Thus the chemical methods frequently cannot be adapted for the detection of penicillin in the small concentrations found in milk.

Methods based on marking antibiotics with dyes

Hargrove et al. (1958) suggested the use of a combination of fat soluble fluorescein (Fluoral) and uranine as

markers to penicillin preparations used for intramammary infusion. The marker which was excreted in proportion to the penicillin could be detected with the naked eye for 48 hr, and with ultraviolet light for 96 hr, after treatment.

Smitasiri _et_ _al_. (1958) used fat-soluble chlorophyll for this purpose. The green color was present in the milk for 5-10 milkings with penicillin. The use of this material appeared quite satisfactory.

Shahani (1958-1959) used a turkish green coloring matter in combination with penicillin, oxytetracycline, chlortetracycline, streptomycin and polymyxin. The results of user trials were also fully satisfactory. This worker (1960) also found that the food dye green 3 could be used successfully as a marker for the detection of neothion in milk.

Dawson (1960) has reported that Brilliant Blue C.F.C., a food dye, has suitable properties for incorporating with penicillin.

It has been suggested that penicillin preparations should be made with an isotope tracer which would make it possible to detect penicillin in milk from treated udders with considerable certainty. Such a test would, however, put unduly great demands on the equipment of an ordinary laboratory (Storgards & Mohr, 1962).

Microbiological methods

Methods based on acid production or redox potential. Silverman & Kosikowski (1952) measured the acid increased by starter organisms in an unknown milk and compared it with the acid increase of a control milk under comparable incubation and testing conditions. If the total activity values (acidity increase of test milk/acidity increase of control milk) x 100, is below 80%, the presence of inhibitory substance is presumed and the milk is submitted to more specific tests.

Ruehe (1950) suggested that milk might be tested for its starter making qualities by heating 10 ml samples in test tubes to 175°F for 5 min, cooling to 72°F and incubating with 1% of starter. Satisfactory milk would coagulate in 10 hr or less at 72°F.

Collins (1957) stated that when heated milk samples were inoculated with Streptococcus thermophilus and incubated at 104°F for 16 hr, those samples which did not coagulate were presumed to contain inhibitors.

Berridge (1956) determined the concentration of penicillin by mixing the milk sample with an actively growing culture of S. thermophilus and comparing the acidity with control mixtures containing known concentration of penicillin. Bromocresol purple was used as the indicator and penicillinase was used to identify penicillin. By using this method, 0.005-0.01 iu of penicillin/ml milk could be measured in 2½ hr.

When a modification of the technique was used 0.015-0.06 iu of penicillin/ml milk could be detected in half an hour.

Neal & Calbert (1955) used 2, 3, 5 triphenyltetrazolium chloride (TTC) to test for antibiotics in milk. The reaction was characterized by a color change from the leucoform to red in the presence of growing bacteria and this conversion was inhibited by low concentrations of antibiotic. A test milk sample was inoculated with a 12-14 hr old culture of _S. thermophilus_ or a commercial lactic starter and incubated for 2 hr at 37°. After the incubation period the solution of TTC was added to the sample which was then incubated for an additional period of 30 min. Control milk samples containing a series of known concentrations of antibiotic were treated the same as the test milk sample. Concentrations of 0.04 iu/ml of penicillin, 0.20 µg/ml of chlortetracycline, 0.5 µg/ml of oxytetracycline and 4.20 µg/ml of streptomycin could be detected by using _S. thermophilus_ as the test organism . Concentrations of 0.3 iu/ml of penicillin, 0.2 µg/ml of chlortetracycline, 1 µg/ml of oxytetracycline and 0.6 µg/ml of streptomycin could be detected by using commercial lactic starter.

Igarashi _et al_. (1960) also used TTC as a test indicator. The milk to be tested was inoculated with 0.5 ml of an actively growing culture of _Bacillus stearothermophilus_ at 61-62°. After 20 min incubation, 1% of TTC solution was

added and incubated for an additional 10-20 min. The samples were cooled and then compared with controls containing known concentrations of penicillin. This test was capable of detecting 0.005 iu of penicillin/ml of milk in 30-40 min.

Schipper (1951) described a method for detecting antibiotic in milk by using methylene blue and B. cereus var. mycoides. With this method 0.016 μg/ml of chlortetracycline/ml of milk could be detected within 4 hr.

Prouty (1960) described a resazurin reduction method for the detection of inhibitory substances in milk. Milk samples were poured into the tubes containing measured quantities of resazurin. The tubes were heated in flowing steam for 5-10 min and cooled at once to 100°F or lower. The tubes were then inoculated with S. thermophilus and incubated at 102-104°F for 2 hr. During the last 30-40 min of the incubation period, the degree and time of dye reduction were recorded. Control samples of milk with known concentrations of penicillin were treated the same as milk samples. The test was reported as being sensitive to 0.01 iu of penicillin.

Microscopical methods. Whitehead & Cox (1956) reported that 0.1 iu of penicillin/ml of milk might be detected by its effect on the morphology of a strain of S. cremoris ML_1 grown in the milk for 5 hr.

Liska (1960) also reported a method similar to that developed by Whitehead & Cox. *S. thermophilus* was used as the test organism. Within 1-1½ hr, 0.015 iu of penicillin, 0.01 iu of bacitracin, 0.15 µg of oxytetracycline, 0.15 µg of chlortetracycline and 0.75 µg of chloramphenicol/ml of milk could be detected.

Disc assay methods. Silverman & Kosikowski (1952) used 0.25 in. diameter filter paper discs saturated with test milk. The discs were placed on an agar surface in a Petri dish previously seeded with a *B. subtilis* spore suspension. The plate was inverted and incubated at 37° for 4-6 hr. The development of a circular inhibition-zone around the disc indicated the presence of antibiotics in the sample. Several discs containing known concentration of antibiotics were treated in the same way as the test sample, and the concentration of antibiotics of the test sample could be deduced by comparison.

The disc assay method listed in Standard Methods for the Examination of Dairy Products (1953) is similar to the method described by Silverman & Kosikowski except that the incubation temperature is 35° instead of 37°.

Gogas & Bicknell (1953) used a 48 hr broth culture of *B. subtilis* as the test organism. Seeded plates were incubated for 2½ hr. The plates could be removed from the incubator after 2½ hr and stored in the refrigerator until

they were needed. By this method, the time required for the detection of penicillin was reduced to 2 hr.

Cerny & Morris (1955) modified the disc assay method by using two 0.5 in. diameter filter paper discs pressed one on top of the other on the surface of the agar. The results were sensitive and precise at least to 0.01 iu of penicillin/ml of milk. Difco dehydrated whey agar and a B. subtilis spore suspension were used in this method.

Hibbs & Royd (1957) demonstrated that the Difco bioassay kit could not detect any antibiotic other than penicillin. Milk samples could be satisfactorily stored in a frozen condition up to 2 weeks without influencing the test for antibiotics.

Pital *et al*. (1956) developed a disc assay method based on the inhibition of microbial reduction of resazurin by antibiotics as a screening test for the selection of an antibiotic of choice for the treatment of disease. Discs containing antibiotic were placed on the surface of seeded plates which were incubated for a short time at 37°. After incubation, the discs were removed and resazurin solution was added to each of the plates which were rotated by hand until the entire surface was covered with the dye solution. After the dye had diffused into the agar, the entire surface of the plate was covered to a depth of several mm with heavy liquid mineral oil. The plates were then incubated at 37°

and observed periodically for color change.

Shahani & Badami (1958) described a modification of the technique developed by Pital et al. (1956). This method also involved flushing the agar in the disc assay method with resazurin. The presence of antibiotic in milk was revealed by the retention of a red or purple zone in the plate. Either Staphylococcus aureus or Lactobacillus bulgaricus was used as the test organism. With this test 0.04 ppm (0.066 iu) of penicillin, 0.04 ppm chlortetracycline, 0.05 ppm oxytetracycline or 0.06 ppm achromycin and 0.17 ppm of streptomycin/ml of milk could be detected in 1.6-1.9 hr.

Igarashi et al. (1960) described a rapid antibiotic assay method using B. stearothermophilus as the test organism. The plates were incubated at 61-62° for 75 min.

Following the suggestion of Igarashi et al. Galesloot & Hassing (1962) used B. calidolactis as the test organism. An active culture was obtained by incubation at 55° for 17 hr. With this method it was possible to detect 0.0025 iu/ml of penicillin in 2½ hr.

Kosikowski and Ledford (1960) described a reverse-phase disc assay test for detecting antibiotics in milk to provide flexibility in field testing for antibiotics. Storage temperature did not exert a great influence on the seeded agar

plates because the nutrients were excluded from the agar and deposited on the test discs instead. In this method using B. subtilis spores, 0.03 iu of penicillin/ml of milk could be detected in 4-6 hr.

Kosikowski & Mocquot (1956) used vacuum freeze-dried milk tablets made of test milk instead of the paper discs. This resulted in an increase in sensitivity of about ten-fold.

Kennedy & Harper (1960) reported an approach to a rapid test for antibiotics in milk. The method was based on the observation that a given number of bacteria of S. cremoris, reduced TTC at a greater rate when the bacteria were packed together. Concentration of 0.1 µg or more of oxytetracycline/ml of milk could thus be detected in 20-30 min.

Kosikowski (1957) suggested a method of controlling the growth of test bacteria for antibiotic assay through anaerobiosis. Sarcina lutea and Micrococcus pyogenes both of which species display aerobic characteristics were used as test organisms. Anaerobiosis was induced either by the removal of air from the environment by high vacuum or by saturating the environment with nitrogen. Three ml of melted sterile nutrient agar, seeded with the test organism, were layered in a disposable plastic Petri dish. After solidification of the agar, the seeded plate was inserted into an aluminum-polyethylene pouch with low oxygen-transmission characteristics. The test organisms under anaerobiosis

remained dormant but survived for 12 days at 34° and on opening the packages were unimpaired. Thus tests could be carried out directly without seeding the plates although growth was less dense and rapid than in unstored plates.

Arret and Kirshbaum (1959) described a rapid disc assay for detecting penicillin in milk. The medium seeded with B. subtilis ATCC 6633 were held at 15° for not less than three or more than five days before they were used. This modification was said to enable detection of 0.05 iu of penicillin/ml of milk in 2½ hr.

EXPERIMENTAL AND RESULTS

Comparison of Tests for Antibiotics in Milk

Comparative tests were made of the three types of methods of testing for antibiotics in milk in order to judge which method might be most suitable for modification and improvement.

Methods

I. Triphenyl tetrazolium chloride reduction method. For this method 8 ml of sterile skim milk and 1 ml of a known concentration of antibiotic were pipetted into a sterile test tube with a screw cap and heated at 80° (176°F) for 5 min. For a control, 8 ml of skim milk and 1 ml of distilled water were placed in a second tube and similarly heated. The tubes were cooled to 37° and inoculated with 1 ml of a 14 hr actively growing culture of *S. thermophilus* NIRD489 diluted 1:1 in sterile skim milk.

The tubes were stoppered and inverted twice to mix the contents and were then incubated in a 37° water bath for 2 hr. Following this, 0.3 ml of a 1:25 solution of 2, 3, 5 triphenyl tetrazolium chloride (TTC) (Eastman Organic Chemicals, Rochester, New York) was added to each tube and inverted twice to mix. The tubes were returned to the water bath, incubated for an additional 30 min, removed and the

change of color compared with the control. A test sample with a distinctly lighter color (less red) than the control was considered to contain an inhibitor.

II. Disc assay method. Bacto whey agar (Difco) was rehydrated by suspending 4.0 g in 100 ml of cold distilled water and heating to boiling to dissolve the medium completely. The medium was sterilized in an autoclave at 121° for 15 min. The sterile medium was cooled to 50-55° and the contents of one ampoule of *B. subtilis* ATCC 6633 spore suspension (Difco) were added to 100 ml of medium. The seeded medium was mixed well avoiding air bubbles, and 10 ml was poured into sterile 15 x 90 mm Petri dishes and allowed to solidify. As soon as the inoculated whey agar had solidified, the blank filter paper discs (0.25 or 0.5 in. diameter) saturated with known concentrations of antibiotics in milk were placed 2-3 cm apart on the surface of the solidified agar. The plates were incubated at 37° for $2\frac{1}{2}$-3 hr. After incubation, the diameter of the inhibition-zones were measured.

III. Microscopical method. For this method 8.8 ml quantities of sterile skim milk (10% w/v) of powdered skim milk (Difco Laboratory, Detroit, Michigan) were pipetted into separate sterile screw cap test tubes and 1 ml of various strengths of the following antibiotics, bacitracin, dihydrostreptomycin sulfate U.S.P., neomycin sulfate, penicillin "G" U.S.P. sodium (Nutritional Biochemicals Corp., Cleveland, Ohio) was added

to each tube except the control in which 1 ml of distilled water was added. The tubes were heated at 80° (176°F) for 3 min and then cooled to 37°. A 14 hr actively growing culture of S. thermophilus NIRD489 was diluted 1:10 with sterile skim milk and held at 37° for 10 min. To each tube, including the control, 0.2 ml of the diluted culture was added to give a total volume of 10 ml/test. The tubes were inverted five times to mix the contents and were then incubated in a water bath at 37° for 60-90 min. At 30 min intervals, each test tube was inverted three times. At the end of the incubation period, the test samples and controls were shaken according to the direction given for shaking dilution blanks for the standard plate count (Standard Methods, 1953). A milk film for each test sample was made by smearing 0.01 ml of test sample over 1 cm^2. The films were stained with the one dip Newman Lampert technique (Laboratory Manual, 1959). The stained milk films were examined under the oil immersion objective using a microscope with a factor of 300,000-500,000. The bacterial clumps were counted in five fields at well separated points on the stained films, after checking for abnormal distortion or enlargement the bacterial cells. Any sample which caused cell distortion or enlargement of the test culture, or has a clump count/field less than 50% of the clump count of the control, was considered as having antibiotic present.

Results

The results from the dye reduction, disc assay and microscopical methods are shown in Table 2.

I. The TTC reduction method. No color change was observed with this test after the dye had been added even though the incubation period of 30 min was extended to 90 min. Accordingly observations were made at 120 min. This method was sensitive to 0.05 iu/ml of bacitracin and 0.01 iu/ml of penicillin but no effect was observed with the samples containing streptomycin and neomycin.

This method was the most sensitive method among these three assay methods. Although 0.01 iu of penicillin/ml of milk can be detected, it took about 4 hr to complete the test. As an actively growing test organism is required with this method it is probable that the age and quantity of the culture used would have some influence on the reproducibility of the method.

II. The disc assay method. The inhibition-zones with this test appeared after 2½ hr incubation but were not sufficiently clear for measurement. An additional 30 min incubation was required to yield an adequately defined inhibition-zone. This method was only sensitive to concentrations of penicillin down to 0.05 iu/ml of milk. Bacitracin, streptomycin and neomycin could not be detected. Positive identification of penicillin can be obtained by the use of "Penase" discs containing penicillinase.

Table 2. Comparison of three methods for microbiological assay of different antibiotics in milk

Antibiotic and concentration	TTC reduction	Disc assay	Microscopic	
	Dye reduction at 120 min incubation at 37°	Diameter of inhibition-zone	Average clump count per field with incubation times of:	
		(cm)	60 min	90 min
Control	R	0	3.80	9.00
Bacitracin				
1.000 iu/ml	NC	0	0.60	0.80
0.500 "	NC	0	0.80	1.00
0.100 "	NC	0	0.40	0.80
0.050 "	NC	0	0.60	0.60
0.020 "	-	-	0.80*	1.20*
0.015 "	-	-	1.00*	0.80*
0.010 "	R	0	1.00*	1.40*
Streptomycin				
1.000 µg/ml	R	0	1.20	4.00
0.750 "	-	-	1.40	4.00
0.500 "	R	0	1.40	4.80
0.100 "	R	0	1.80	6.40
0.050 "	R	0	2.20	5.80
0.010 "	R	0	1.80	3.60
Neomycin				
1.000 µg/ml	R	0	1.40	3.40
0.500 "	R	0	1.40	1.50
0.100 "	R	0	1.60	2.80
0.050 "	R	0	1.00	4.20
0.010 "	R	0	0.20	4.60
Penicillin				
1.000 iu/ml	NC	1.85	1.40	1.20
0.500 "	NC	1.70	0.60	0.60
0.100 "	NC	1.10	1.20	0.60
0.050 "	NC	0.95	0.80*	0.40*
0.020 "	-	-	1.00*	0.80*
0.015 "	-	-	1.00*	1.00*
0.010 "	NC	0	1.60*	1.20*

*	cells swollen
R	dye reduced
NC	dye not reduced
-	not tested

The disc assay procedure is simple. A standardized B. subtilis spore suspension can be kept under refrigeration for months. The zone of inhibition in a positive test is easily observed and the approximate amount of penicillin may be measured by comparison with known additions of the antibiotic. Penicillin may be positively identified, but for antibiotics other than penicillin, the method suffers from lack of sensitivity and usually it is unable to detect even 1.0 iu/ml of milk. The sensitivity of the disc assay method for penicillin is lower than that of the TTC reduction assay method. Only concentration of 0.05 iu/ml penicillin or above can be detected, rendering the test not useful for the low concentration of penicillin usually found in market milk and milk products.

Galesloot (1962) has observed that paper discs sometimes contain inhibitory substances which are not distributed regularly over the paper; fortunately the false positive results can easily be recognized. Similar cases have been observed in this laboratory. The false positive results were easily distinguished and repeated examination identified that these were due to inhibitory substances contained in the discs.

III. Microscopical method. The results in Table 2 indicate that the microscopical method was not sufficient reliable for effective measurement of different concentrations of antibiotics. There was no apparent cell distortion or

enlargement in the test samples containing streptomycin and neomycin. Swollen cells were found in the test samples containing 0.01-0.02 iu/ml of bacitracin and 0.01-0.05 iu/ml of penicillin.

This method seemed impracticable because it was difficult to distinguish slight morphological changes of test organisms in comparison with the control. In addition errors could easily occur in the clump count because the test organism being a streptococcus does not lend itself well to the clump count as the chains are readily broken when the samples are shaken to distribute the organisms evenly. For instance, the average clump obtained in the samples containing 0.01 µg/ml streptomycin were less than 50% of average clump in control, whereas the average clump in the samples containing 0.05 µg/ml streptomycin were higher. Similar anomalous results were obtained on other separate occasions and the test in this laboratory did not correspond with those reported by Liska (1960). In his report, the minimum concentration of various antibiotics detected were: penicillin 0.015 iu/ml and bacitracin 0.01 iu/ml of milk; the average clump counts in controls after 60 and 90 min incubation were approximately 10 and 20 micro-organisms respectively. While the test organism used in this work did not behave in the same way as the culture used by Liska, it must be admitted that this was not the same culture. However

previous work with this test using the identical culture used by the originators of the test (Whitehead & Cox, 1956) failed to give a result in under 5 hr. This suggested that the culture might have change somewhat; hence the use of a different culture.

Attempts to Improve the Sensitivity of Assay Methods for Penicillin in Milk

From the foregoing it appeared that the microscopical test was not an easily reproducible test for routine purposes and accordingly attemps to improve the sensitivity of tests for antibiotics in milk were confined to the TTC reduction and disc assay tests. Six methods were tried out to improve the sensitivity of these two tests and the results of this investigation are given below.

I. Cobalt chloride

This method was suggested by Pratt & Dufrenoy (1948) who found this compound enhanced the action of penicillin from among many compounds tested. They suggested that the effectiveness of cobalt in lowering the threshold concentration of penicillin for bacteriostasis *in vitro* and possibly *in vivo* may be ascribed to formation of complexes involving sulfhydryl groups associated with some enzymes.

Reconstituted skim milk (Difco) was divided into four, 250 ml quantities; to each, cobalt chloride and penicillin "G"

sodium (Nutritional Biochemicals Corp., Cleveland, Ohio) were added in varying concentrations (see Tables 3 & 4). The milk samples were heated at 82.2° (180°F) for 5 min and cooled to room temperature. The disc assay method modified by Cerny & Morris (1955), using 6 ml of seeded agar and the double filter paper discs (0.5 in. diameter) and the TTC reduction method were used in this study.

The results are presented in Tables 3 & 4. These show there was no observable effect as a result of the addition of cobalt to the test samples.

Table 3. The effect of varying additions of cobalt chloride on the activity of penicillin in milk with the TTC reduction method

Concentration of penicillin (iu/ml)	Reduction of TTC in milk with different additions of cobalt chloride (mg/liter)			
	0	1	10	100
0	R	R	R	R
0.001	R	R	R	R
0.003	R	R	R	R
0.005	R	R	R	R
0.010	NC	NC	NC	NC

R dye reduced
NC dye not reduced

Table 4. The effect of varying additions of cobalt chloride on the activity of penicillin in milk with the disc assay method

Concentration of penicillin (iu/ml)	Diameter of inhibition-zone (cm) with different additions of cobalt chloride (mg/liter)			
	0	1	10	100
0	0	0	0	0
0.01	0	0	0	0
0.02	1.5	1.5	1.5	1.5
0.05	2.0	2.0	2.0	1.9
0.10	2.2	2.1	2.2	2.1

II. Surface active agents

The addition of surface active agents was suggested by Bruce & Mitchell (1952) who thought that these compounds might promote the diffusion of the antibiotics through the test medium and render the cell wall more susceptible to the action of the antibiotic.

The surface active agents used were Tween 80 (polyoxyethylene sorbitan monooleate), Tween 20 (polyoxethylene sorbitan monolaurate) (Atlas Powder Co., Canada); Duponol WA

powder (lauryl sodium sulfate) (Candian Industries Ltd.) and Triton x-100 (alkyl aryl polyether alcohol) (Rohn & Haas Co., Pa.).

A batch of reconstituted skim milk (Difco) was divided into 200 ml quantities, each containing one of the above surface agents in concentrations 0, 40, 100 and 200 μg/ml. Sub-samples of 20 ml were prepared to which penicillin "G" sodium ranging from 0.001-0.10 iu/ml was added. The samples were heated at 82.2° for 5 min and cooled to room temperature.

The disc assay method modified by Cenry and Morris (1955) and the TTC reduction method were used in this study.

The results are given in Tables 5 & 6 and show that the four surface active agents used had little apparent effect in enhancing the potency of penicillin against S. thermophilus and B. subtilis in the TTC reduction or disc assay methods.

III. X-ray irradiation

This was suggested by Thatcher (1962). It is known that X-ray can cause mutations in subsequent generations of irradiated micro-organisms and it was hoped to yield penicillin sensitive mutants by this procedure.

S. thermophilus NIRD489 and B. subtilis ATCC 6633 were irradiated in the Microbiology Section, Food & Drug Directorate, Department of National Health & Welfare, Ottawa, as follows:

Table 5. The effect of the addition of surface active agents to penicillin against *Streptococcus thermophilus* in the TTC reduction method

Concentration of penicillin (iu/ml)	Reduction of TTC in milk with different additions of various surface active agents as follows:												
	0	40 µg/ml				100 µg/ml				200 µg/ml			
		Tween 80	Tween 20	Duponol	Triton	Tween 80	Tween 20	Duponol	Triton	Tween 80	Tween 20	Duponol	Triton
0	R	R	R	R	R	R	R	R	R	R	R	R	R
0.001	R	R	R	R	R	R	R	R	R	R	R	R	R
0.003	R	R	R	R	R	R	R	R	R	R	R	R	R
0.005	R	R	R	R	R	R	R	R	R	R	R	R	R
0.010	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

R	dye reduced
NC	dye not reduced

Table 6. The effect of the addition of surface active agents to penicillin against *Bacillus subtilis* in the disc assay method

Concentration of penicillin (iu/ml)	Diameter of inhibition-zone (cm) with different additions of surface active agents												
	0	40 µg/ml				100 µg/ml				200 µg/ml			
		Tween 80	Tween 20	Duponol	Triton	Tween 80	Tween 20	Duponol	Triton	Tween 80	Tween 20	Duponol	Triton
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.01	0	0	0	0	0	0	0	0	0	0	0	0	0
0.02	1.50	1.50	1.50	1.50	1.45	1.50	1.50	1.50	1.50	1.50	1.45	1.50	1.50
0.05	1.95	1.95	1.85	1.95	1.85	1.95	1.90	1.85	1.90	1.90	1.85	1.90	1.90
0.10	2.10	2.10	2.10	2.00	2.00	2.10	2.00	2.00	2.00	2.05	2.00	2.10	2.05

Cultures were transferred to nutrient broth containing 0.3% yeast extract (+1.0% sucrose for *S. thermophilus*) and following 24 hr incubation at 35.5° for *B. subtilis*, and 43° for *S. thermophilus*, the broth suspension was used to inoculate 100 ml of fresh broth which was incubated as above for another 24 hr. Following this, the broth was well shaken and dispensed in 10 ml quantities in vials for irradiation.

The irradiated *S. thermophilus* was transferred to skim milk for the TTC reduction method and the irradiated *B. subtilis* was prepared as a spore suspension for the disc assay method.

B. subtilis spores were produced in Roux bottles containing 300 ml of antibiotic medium 1 (Difco) and magnesium sulfate in a concentration of 300 mg/liter of medium. The surface of the agar in the Roux bottle was inoculated with a spore suspension of *B. subtilis* from a 24 hr culture and then incubated at 37° for 5 days. The resulting growth was harvested in 50 ml of sterile 0.85% sodium chloride solution. The suspension was shaken for 2½ hr in a bottle containing glass beads and the resultant crude suspension was heated at 70° for 30 min to destroy vegetative organisms and to favor the germination of spores. The suspension was centrifuged at approximately 1,500 rev/min for approximately 1 min to throw down any remaining clumps and large pieces of debris. The sediment was discarded and the supernatant containing the

spores was centrifuged at 3,000 rev/min for 40 min to throw down the spores. The supernatant was decanted and discarded after microscopic examination had revealed the absence of spores. The sediment of spores was suspended in a small volume of sterile saline by mechanical shaking. The volume of suspension in each tube was adjusted with sterile saline to approximate two-thirds of the capacity of the tube. The contents were centrifuged and washed similarly 5 times. The spore suspension was diluted with saline to a reading of 264 with No.47 filter (445-505 mμ) on the Klett-Summerson Colorimeter (Klett Manufacturing Co., New York).

The effect of X-ray irradiation on the test organisms was examined by the TTC reduction and disc assay methods.

Table 7. The effect of irradiated <u>Streptococcus thermophilus</u> used as the test organism in the TTC reduction method

Concentration of penicillin (iu/ml)	Doses of irradiation (rep)			
	0	50,000	250,000	450,000
0	R	R	R	R
0.001	R	R	R	R
0.004	R	R	R	R
0.007	R	R	R	R
0.010	NC	NC	NC	NC

R dye reduced
NC dye not reduced

Table 8. The effect of irradiated <u>Bacillus subtilis</u> used as the test organism in the disc assay method

Concentration of penicillin (iu/ml)	Diameter of inhibition-zone (cm) with the following doses of irradiation (rep):			
	0	50,000	250,000	450,000
0	0	0	0	0
0.01	hazy	hazy	hazy	hazy
0.02	1.90	1.80	1.90	1.95
0.03	2.00	1.90	2.15	2.10
0.04	2.10	2.00	2.10	2.10
0.05	2.25	2.15	2.20	2.20
0.10	2.35	2.30	2.35	2.30

The results shown in Tables 7 & 8 indicate that the sensitivity of the test organisms, <u>S. thermophilus</u> and <u>B. subtilis</u>, to penicillin was not apparently increased by X-ray irradiation in either the TTC test or the disc assay method. In the latter, the inhibition-zone which appeared after 3 hr of incubation was not sufficiently clear though after 4 hr of incubation the zones were bigger than in previous trials in which standard spore suspension (Difco) had

been used. It is believed that there were fewer spores in the spore suspension prepared in this laboratory than in the Difco spore suspension, and that this accounted for the delay in the formation of the inhibition-zone and in consequence its increase in size.

The mutation rate of B. subtilis subjected to X-ray irradiation has been reported as 3-6% by Burkholder & Giles (1947), therefore the isolation of mutants after irradiation would be necessary for any change in sensitivity to be observed. The replica plating technique developed by Lederberg & Lederberg (1952) can be used for the purpose. No work has been attempted here to isolate mutants with sensitive characteristics to penicillin as it was considered that the work involved might be too great, particularly when there was no assurance of success.

IV. Vacuum freeze-drying

This was suggested by Kosikowski & Mocquot (1956). They have found that antibiotic in milk may be concentrated by vacuum freeze-drying. A round compressed milk tablet made from the milk containing a known concentration of antibiotic and used instead of paper in the disc assay method has increased the sensitivity of this method about ten-fold. The object of this work was to observe the effect of different sizes of milk tablets and different quantities of seeded agar on the sensitivity of this modification.

A known antibiotic-free milk taken from the University Farm was divided into 11 volumes of 18 ml and to each penicillin "G" sodium was added in concentrations ranging from 0.001-0.01 iu/ml. The milks were then heated at 82.2° for 5 min and cooled to room temperature. Two ml portions of each test milk were pipetted into a special bulbs and freeze-dried (The Vir Tis Co., Inc., Gardiner, New York). By using this apparatus, eleven samples could be prepared in approximately 1½ hr. Each sample of dried test milk was introduced through a funnel into a strong glass tube with the opposite end lying flat against a clean hard surface. The milk powder was compressed with a glass rod, closely fitting the internal diameter of the tube. After two or three poundings with the rod, the milk powder became sufficiently cohesive to be forced out, as a round compressed tablet, onto an agar surface seeded with B. subtilis. The plates were incubated at 37° for 3 hr. A clear zone around a disc indicated the presence of penicillin or other antibiotic. The results of the application of disc assay method with different sizes of freeze-dried milk tablets and different amounts of seeded agar are given in Table 9.

Table 9. The effect on the sensitivity of the disc assay method for penicillin of different sizes of circular dried milk tablets and different quantities of seeded agar

Concentration of penicillin (iu/ml)	Diameter of inhibition-zone (cm) with: 0.9 cm diameter tablet & 10 ml seeded agar	1.3 cm diameter tablet & 6 ml seeded agar
0	0	0
0.001	0	0
0.002	0	0
0.003	0	0
0.004	0	hazy
0.005	hazy	1.40
0.006	1.20	1.45
0.007	1.20	1.50
0.008	1.25	1.55
0.009	1.25	1.60
0.010	1.30	1.70

In the original paper (Kosikowski & Mocquot, 1956), the sensitivity of this method was reported as 0.01 iu/ml of penicillin, whereas in this laboratory, 0.006 iu/ml of penicillin could be detected with a 0.9 cm diameter tablet & 10 ml seeded agar; with a 1.3 cm diameter tablet & 6 ml seeded agar 0.005 iu/ml of penicillin could be detected. The fact that most of the circular dried milk tablets had collapsed by the end of the incubation period as a result of absorbing moisture from the agar, might have increased the sensitivity of the test by bringing the milk tablet into more intimate contact with the agar.

Several other workers have reported that by using larger diameter discs and thin layers of seeded agar the test for penicillin was made more sensitive. The results in Table 9 would seem to confirm this. However the same amount of antibiotic was used here in both sizes of tablet and it would seem therefore that only the thickness of the layer of agar is responsible for the increase in sensitivity as a result of the greater horizontal diffusion of the antibiotic.

V. Milk evaporation

Mention of evaporation of milk as a method of concentrating antibiotics in test samples has not been observed in the literature. As there seemed a reasonable possibility of increasing the sensitivity of tests for antibiotic in milk

by this method, it was investigated.

The apparatus used for evaporation consisted of a hydro-aspirator, a condenser, a water bath and a rotary film evaporator (Rinco Instrument Co., Greenville, Ill., Plate 1). To a hundred ml of a known antibiotic free milk was added penicillin "G" sodium ranging from 0.0017-0.34 iu/ml and heated to 82.2° (180°F) for 5 min. The milk was weighed and then evaporated in the evaporating flask at 55° (131°F) for 20 min. The flask of the rotary film evaporator was rotated as soon as the water bubbles start to break to prevent violently foaming milk being sucked into the vacuum line during evaporation. The evaporated milk sample was weighed again and served as the test sample. The TTC reduction method and the disc assay method were used in this trial.

The effect of heating on the potency of penicillin in milk

There are suggestions in the literature that the effect of heat plays an important role on the potency of penicillin. Foster & Woodruff (1943) pointed out that when aqueous penicillin solutions were heated at 65° for 30 min this resulted in a 5-30% inactivation, whereas Trembath (1950) suggested that there was no effect on the potency of penicillin in milk which had been heated at 68° for 10 min or 88° for 30 min. Marth & Ellickson (1959)have summarized and tabulated the effect of various heat treatment on antibiotics. It is

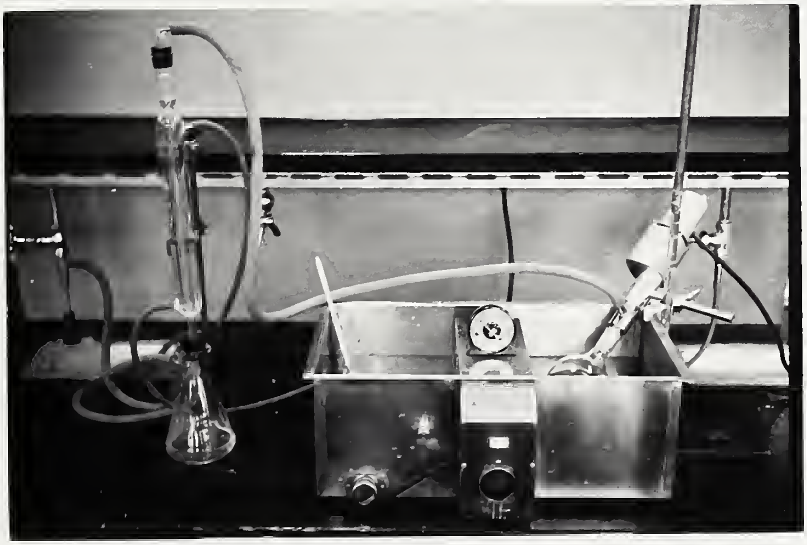

Plate 1. Apparatus used for concentrating antibiotic in test samples of milk and whey.

generally recognized that penicillin, chlortetracycline, oxytetracycline and chloramphenicol in milk are relatively stable to pasteurization treatments and slightly above. Recently, Kosikowski (1963) has reported that penicillin, chlortetracycline, oxytetracycline and streptomycin were stable in milk heated to 180°F for 5 min.

Accordingly tests were made to check whether the heating during evaporation had any effect on the potency of penicillin in milk. To each test tube, 9 ml of sterile rehydrated milk powder and 1 ml of penicillin ranging 0.1-50 iu/ml were added with the exception of the controls. Samples were divided into four lots of 10 tubes each and heated as follows: 55° for 10 min; 82.2° for 5 min; 82.2° for 5 min followed by 55° for 10 min; the control was unheated. The samples were cooled immediately and tested by the disc assay method modified by Cerny & Morris (1955). The results obtained in this laboratory shown in Table 10 have confirmed that penicillin in milk is stable to treatments somewhat above that required for pasteurization of milk, in that preliminary tests (Mei & Clegg, 1962) showed that heating to 55° for 20 min showed only 3% inactivation of penicillin.

Igarashi (1960) used <u>B. stearothermophilus</u> as the test organism and 61-62° as the temperature of incubation, 30-40 min in TTC reduction and 75 min in disc assay method, and Galesloot (1962) used <u>B. calidolactis</u> as the test organism and 55° as the temperature of incubation for 2½ hr.

Table 10. The effect of heat on the potency of penicillin "G" sodium in milk as determined by the disc assay method

Concentration of penicillin (iu/ml)	Diameter of zone (cm) with heat treatment of: Unheated	55° for 10 min	82.2°for 5 min	82.2° for 5 min & 55° for 10min
0	0	0	0	0
0.01	0	0	0	0
0.02	1.50	1.40	1.40	1.50
0.03	1.55	1.50	1.50	1.55
0.04	1.65	1.65	1.70	1.65
0.05	1.80	1.70	1.80	1.80
0.10	1.95	2.00	1.95	1.95
0.50	2.40	2.35	2.40	2.35
1.00	2.50	2.50	2.50	2.50
5.00	2.80	2.80	2.75	2.80

Accordingly, a temperature of 55° used for evaporation should not result in any reduction on the potency of penicillin.

The effect of concentrating penicillin in milk on antibiotic tests

Samples of 100 ml of milk containing known additions of penicillin were evaporated in a rotary film evaporator as described earlier and tested by the TTC reduction and disc assay methods. The results are given in Tables 11 & 12.

Table 11. The effect of concentrating penicillin in samples of test milk by evaporation on the TTC reduction method

Concentration of penicillin added (iu/ml)	Weight of evaporated milk (g)	Approx. concentration of penicillin in evaporated milk (iu/ml)	Dye reduction
0	30.5	0	R
0.0017	30.5	0.0056	R
0.0034	32.0	0.0106	NC
0.0340	32.0	0.1060	NC

R dye reduced
NC dye not reduced

Table 12. The effect of concentrating penicillin in samples of test milk by evaporation on the disc assay method

Concentration of penicillin added (iu/ml)	Weight of evaporated milk (g)	Approx. concentration of penicillin in evaporated milk (iu/ml)	Diameter of inhibition-zone (cm)
0	28.0	0	0
0.017	29.0	0.058	1.75
0.034	28.1	0.120	1.95
0.170	27.1	0.627	2.45
0.340	29.0	1.170	2.60

The results in Tables 11 & 12 show that 100 ml of milk can be evaporated to less than one-third of its volume at 55° and under a vacuum of ca 15 mm Hg in 20 min.

The 3:1 reduction in volume with evaporated milk gave a product which was not too viscous for use with the TTC reduction and disc assay methods.

The sensitivity of the TTC reduction method was greater than disc assay method. The TTC reduction method gave a positive result with the sample containing 0.0034 iu penicillin/ml

whereas the disc assay gave a positive result with the sample containing 0.017 iu penicillin/ml. Greater sensitivity might be obtained by lengthening the time of evaporation with a resultant a greater reduction in volume. However when this was attempted a higher viscosity of evaporated milk resulted causing the test samples to be too viscous to be handled. Although the amount of penicillin in milk may be concentrated more than three times by this technique, this is still not sufficiently sensitive to detect the small amounts of penicillin commonly found in market milk.

VI. Whey concentration

As the increase in sensitivity of the detection of antibiotics by milk evaporation was limited by the viscosity of the sample, it seemed reasonable to determine whether the antibiotic was associated with the milk solids which were interfering with the ease of handling the sample. Accordingly tests were made to determine whether antibiotics were associated with the cream, or the other solids of milk.

Recovery of penicillin from different milk fractions. A known concentration of penicillin was added to sterile distilled water, skim milk and whole milk to determine the relative recovery from these three media. A portion of the whole milk sample was centrifuged at 1,000 rev/min for 5 min to remove the cream. To 100 ml of the whole milk was added 1 ml of cheese rennet which was then placed in a water bath at 40°

for about 10 min to clot the milk. The whey was separated by filtration. These samples were tested by the modified disc assay method of Cerny & Morris (1955) and the results are shown in Table 13.

Table 13. Comparison of the recovery of penicillin in different media by the disc assay method

Penicillin contained in	Inhibition-zone (cm) with penicillin concentrations of: 0.05 iu/ml	0.10 iu/ml
Distilled water	1.80	2.00
Reconstituted skim milk from powder	1.80	2.00
Whole milk	1.80	1.95
Skim from whole milk above	1.80	2.00
Whey from whole milk above	1.80	2.05

Table 13 shows that the recovery of penicillin from all the media is the same. These data do not give any indication as to whether the antibiotic is associated with one or more of the constituents of milk, but this information was not essential to the work. The fact that the concentra-

tion of penicillin remained apparently the same in whole milk and whey prepared from the whole milk, made it possible for a greater concentration of the antibiotic by evaporation than had been possible with whole milk.

Technique of whey concentration. A sample of 100 ml of raw whole or skim milk containing a known concentration of penicillin"G" sodium was heated at 82.2° (180°F) for 5 min and cooled to room temperature to destroy some natural inhibitory substances. Concentrated milk or other heated milk products need no such heating. One ml of cheese rennet was added and the test sample placed in a water bath at 40° for about 10 min to clot the milk. The whey was separated by filtration. Ten ml of whey was pipetted into the rotary film evaporator connected with a vacuum condenser. The whey was condensed at 55° (131°F). A 10:1 reduction in volume could be obtained in about 7 min.

In testing milk powder 30 g of powdered milk was added to 90 ml of sterile distilled water. The preparation of the sample was the same as for fluid milk except that the test samples were not heated.

Difficulty has been encountered in separating the whey from the concentrated and evaporated milk by rennet. It is possible that forewarming exerts its influence through shifts in salt equilibria or through denaturation of the

serum proteins (Jenness & Patton, 1959). The intervention of preheating, condensing, and the addition of stabilizers can alter the salt pattern of milk enormously (Pyne, 1962). Attempts have been made in this laboratory by adding 0.3 ml of concentrated lactic acid into 100 ml of diluted 1:1 evaporated milk and 1 ml of rennet. The flocculation took place in the evaporated milk in a few minutes but an inhibition-zone would appear in 10:1 concentrated whey without adding penicillin. One ml of 0.1 M calcium chloride was added to 100 ml of evaporated milk with 1 ml of rennet but no improvement has been shown. As an alternative to rennet, ultrafiltration was used to separate the whey from evaporated and concentrated milk. This however, lengthened the time necessary to perform the disc assay method.

In testing evaporated milk, 70 ml of evaporated milk was pipetted into a 250 ml capacity cylinder and diluted with 70 ml of distilled water. A hydro-aspirator connected with the ultrafilter (LKB-Produkter AB Sweden) was dipped into the milk. About 12 ml of whey could be obtained within 2 hr.

The degree of concentration of whey in the evaporator is difficult to manipulate precisely although a circular area equal to the volume occupied by 1 ml of whey had been marked on the evaporating bottle to measure the degree of concentration of whey.

The volume of concentrate absorbed by the filter paper discs may be measured accurately by pipetting the concentrated whey to be tested in the disc put previously onto the surface of medium but for routine testing this technique is laborious and cumbersome.

The disc assay method modified by Cerny and Morris (1955) was used in this study. Two more discs were saturated with Bacto-Penase (Difco Laboratories), and placed 2 mm apart from the test sample discs to determine if the inhibitory substance in test sample were penicillin. A partial inhibition-zone indicates that the inhibition is due to penicillin (Plate 4). If a complete inhibition-zone appears, the inhibitory substance is either an antibiotic other than penicillin or one or more other inhibitory substances.

The maximum sensitivity of the modified disc assay method with 6 ml of seeded agar and double disc was found to be 0.02 iu/ml, whereas Cerny & Morris reported a sensitivity of 0.01 iu/ml for this test. The difference might have been caused by the fact that these workers used an 8 hr incubation at 37° whereas in the present work only 3 hr incubation was used: this could have resulted in less diffusion of penicillin in the present work.

Tests with the whey concentration technique. Table 14 shows that 0.003 iu/ml of penicillin in milk could readily be detected by using the modified disc assay method. Greater sensitivity can be obtained by concentrating 20 ml of whey to 1 ml. In this case, an additional 7 min for evaporation would be needed, but this would detect penicillin in a concentration as low as 0.0015 iu/ml (Plate 2). Different concentrations of penicillin "G" (0.001, 0.002 and 0.003 iu/ml) in 0.85% sodium chloride solution were heated at 82.2° for 5 min. Each sample was condensed in the rotary film evaporator at 55° from 10 to 1 ml and then tested with the modified disc assay method. No inhibition-zones were present with the discs containing 0.001 iu/ml of penicillin but zones with an average diameter of about 1.45 cm were observed with samples containing 0.002 iu/ml and zones with diameters of about 1.7 cm were observed with 0.003 iu/ml concentration of penicillin in milk. Partial inhibition-zones appeared beside discs saturated with penicillinase solution but complete inhibition-zones appeared beside discs saturated with sterile saline solution instead of penicillinase solution. These results suggest that penicillin is quite stable to the various heat treatments specified above and 0.003 iu/ml of penicillin in milk can definitely be detected by using the whey concentration technique. Further, it was also revealed in this

test that the activity of penicillin was not inactivated by saline solution.

Table 14. The sensitivity of the whey concentration technique for penicillin in milk as determined by the disc assay method

Concentration of penicillin "G" added (iu/ml)	Diameter of inhibition-zone (cm)
0	0
0.001	0
0.002	1.35
0.003	1.50

This modification has increased the complexity of disc assay method, however, it is easy to run the test in an ordinary laboratory, since the materials used are readily available and the test is capable of detecting 0.003 iu/ml or 100,000 iu of penicillin in 8,000 gal of milk. This modification may be used to trace the small amounts of penicillin in commercial milk and milk products.

Plate 2. A comparison of standard plate assay.

A) 0.02 iu of penicillin/ml added to 1:9 reconstituted skim milk powder with the modified procedure;
B) 20:1 whey concentrate from a 1:9 reconstituted skim milk powder to which 0.0015 iu/ml penicillin had been added; and
C) a 10:1 whey concentration from 1:9 reconstituted skim milk powder containing 0.003 iu/ml of penicillin.

The Application of the Whey Concentration Technique to Commercial Milk and Milk Products

The object of this work was to make use of the whey concentration technique to check whether samples of market milk contain small amounts of penicillin. A small scale survey for the presence of residual penicillin in pasteurized milk, evaporated milk and milk powder was undertaken. The results are shown in Tables 15 & 16.

Three brands of pasteurized milk, "Palm" (Palm Dairies Ltd.), "Nu-Maid" (Northern Alberta Dairy Pool) and "Silverwood's" (Silverwood Dairies Ltd.) were sampled twice a week from food stores in south Edmonton. The powdered skim milks tested were "Alpha" skim milk (Alpha Co., Red Deer, Alta.), "Mil-Ko" (Mil-Ko Products Ltd., Hamilton, Ont.), "Pacific" (Pacific Milk Division, Fraser Valley Milk Producer's Association, Vancouver, B.C.), "Carnation" (Carnation Co., Toronto, Ont.), "Lucerne" (Canada Safeway Ltd., Winnipeg, Man.), "Borden's" (The Borden Co., Toronto, Ont.) and "Kraft", instant powdered whole milk (Kraft Foods, Chicago, Ill.). The evaporated milks tested were "Pacific", "Alpha", "Lucerne" and "Carnation" produced by the companies as stated above (except that the Carnation milk was produced at Wetaskiwin, Alta.), and "Farmer's Wife 1" (Cow & Gate (Canada) Ltd., Brockville,

Ont.). The concentrated milks tested were "Farmer's Wife" Red Band, "Farmer's Wife 2", "Farmer's Wife 3", "Pacific", and "Morning" (Carnation Co.). The method applied was the same as described under the whey concentration method except that the test samples were not heated to 82.2° for 5 min.

In Table 15, the results show that no penicillin positive sample was found in "Palm" milk. Four samples were found containing penicillin out of 18 samples tested (22.2%) in "Nu-Maid" milk, and three positive tests out of 18 tested samples (16.6%) in "Silverwood's". Only one sample of "Nu-Maid" milk containing approximately 0.10 iu/ml of penicillin in fluid milk could be detected by the modified disc assay, however, the inhibition-zones obtained by using whey concentration technique were invariably larger. Since some test samples did not have an inhibition-zone, and some had shown positive results with negative penicillinase identification, some of the samples must have contained inhibitory substances other than penicillin.

In Table 16, the results show that three brands of powdered milk, "Mil-Ko", "Pacific" and "Kraft" were found containing small quantities of penicillin but no penicillin positive sample was found in evaporated milk or concentrated milk samples tested.

Table 15. Results of application of the whey concentration technique in detecting penicillin in commercial samples of pasteurized milk (1963)

	"Palm"				"Nu-Maid"				"Silverwood's"			
	Inhibition-zone diameter (cm)		Penicillinase identification	Approx. penicillin concentration (iu/ml)	Inhibition-zone diameter (cm)		Penicillinase identification	Approx. penicillin concentration (iu/ml)	Inhibition-zone diameter (cm)		Penicillinase identification	Approx. penicillin concentration (iu/ml)
Date	Fluid milk	Concentrated whey			Fluid milk	Concentreated whey			Fluid milk	Concentreated whey		
Jan.												
19	0	hazy	-	0	0	1.40	-	0	0	hazy	-	0
23	0	hazy	-	0	0	hazy	-	0	0	hazy	-	0
25	0	0	-	0	0	0	-	0	0	0	-	0
29	0	1.75	-	0	0	0	-	0	0	0	-	0
Feb.												
1	0	1.65	-	0	0	1.40	-	0	0	1.70	+	0.004
5	0	1.40	-	0	0	0	-	0	0	2.15	+	0.020
8	0	1.45	-	0	0	1.60	+	0.003	0	hazy	-	0
12	0	hazy	-	0	0	1.40	-	0	0	hazy	-	0
15	0	1.45	-	0	0	1.40	-	0	0	hazy	-	0
19	0	1.55	-	0	0	1.80	+	0.005	0	1.50	-	0
22	0	1.75	-	0	1.55	2.60	+	0.100	0	0	-	0
26	0	1.55	-	0	0	hazy	-	0	0	hazy	-	0
Mar.												
1	0	1.50	-	0	0	1.70	+	0.004	0	0	-	0
5	0	1.80	-	0	0	0	-	0	0	0	-	0
8	0	1.90	-	0	0	hazy	-	0	0	1.90	+	0.010
12	0	1.70	-	0	0	0	-	0	0	0	-	0
15	0	1.60	-	0	0	0	-	0	0	0	-	0
19	0	1.55	-	0	0	hazy	-	0	0	0	-	0

[illegible]

([illegible])

[illegible]

Table 16. Results of testing commercial samples of powdered milk, evaporated milk and concentrated milk for penicillin by the whey concentration technique

Brand	Diameter of inhibition-zone (cm)	Penicillin-ase identification	Approximate penicillin concentration (iu/g)
<u>Powdered milk</u>			
"Alpha"	hazy	-	0
"Mil-Ko"	1.5	+	0.006
"Pacific"	1.6	+	0.009
"Carnation"	hazy	-	0
"Lucerne"	0	-	0
"Borden's"	hazy	-	0
"Kraft"	1.8	+	0.015
<u>Evaporated milk</u>			
"Pacific"	0	-	0
"Alpha"	0	-	0
"Lucerne"	hazy	-	0
"Carnation"	0	-	0
"Farmer's Wife 1"	hazy	-	0
<u>Concentrated milk</u>			
"Farmer's Wife" Red Band	hazy	-	0
"Farmer's Wife 2"	0	-	0
"Farmer's Wife 3"	1.7	-	0
"Morning"	0	-	0
"Pacific"	0	-	0

The results shown in Tables 15 & 16 suggest that some milk plants did not conscientiously carry out the test for antibiotics in milk and that the existing methods used are not sensitive enough to detect low concentrations of penicillin in milk; further the zero tolerance mainly depends upon the sensitivity of assay method used.

Kosikowski (1963) tested 85 samples of raw, fresh milk during a 2 years period which were known to be antibiotic free but which gave positive zones of inhibition with the disc assay. All these samples lost their zone-forming ability after being heated to 180°F for 5 min. Thus, natural inhibitory substances probably do not persist in processed milk after preheating. Therefore the positive-zoning in the present survey must have been caused by antibiotics other than penicillin or other chemical substances.

Plate 3. Test for penicillin in "Kraft" instant whole milk powder.

D) 1:3 reconstitution;
E) whey separated from D;
F) 10:1 concentration of E (milk powder containing approximately 0.015 iu/g penicillin).

Plate 4. Identification of penicillin with standard and modified tests.

G & H) 1:9 reconstituted skim milk powder to which 0.05 iu penicillin/ml had been added, showing effect of penicillinase disc K;

I & J) 10:1 whey concentration from 1:3 reconstitution of "Kraft" instant whole milk powder containing approximately 0.015 iu/g penicillin showing similar action of penicillinase disc K.

GENERAL DISCUSSION

A rapid speedier test for detecting antibiotics in milk would clearly be desirable. With such a test it would be possible to reject milk on the dairy platform for the presence of antibiotic. None of the tests in the literature are sufficiently rapid for a rejection test, and the only test that approaches the time required for a rejection test is that devised by Berridge (1956). This is a thirty-minute test but it is elaborate and requires the maintenance of a bacterial culture in the logarithmic growth phase at all times. The simpler tests such as the TTC dye reduction test or the disc assay test both require two and a half hours before a positive result can be read; this is too long for a rejection test.

None of the tests examined in this work, or the modifications made to these tests, resulted in a more rapid test being developed. It has however been possible to devise more sensitive tests and, although these are not as useful as more rapid tests, they are still desirable. Such sensitive tests probably have no value as screening tests because they are admittedly somewhat more cumbersome, but as confirmatory tests for milk supplies in bulk i.e. raw bulk milk awaiting processing, pasteurized milk, condensed milk, evaporated milk

or powdered milk, such tests should be of value.

It is not easy to see how a more rapid test could be devised. Chemical tests appear to be too insensitive and thus we must rely on a biological test. Micro-organisms take time to grow and any tests which rely on an observation of growth or on the effect of growth, must have an inherent waiting period. There would appear to be no advantage in attempting to speed up the test merely by increasing the number of micro-organisms. If for example the number of spores used in seeding the disc assay plates were increased this would increase the number of resistant cells which would be able to grow in the zone of inhibition and thus partially obscure it. On the other hand, if smaller numbers of micro-organisms were used one could expect a smaller number of resistant cells and consequently less likelihood of the zone of inhibition being obscured but then the zone would probably be less definite and less easy to observe because of the sparser bacterial growth. Similarly, increasing the inoculum in the TTC dye reduction test might appear at first thought to carry possibility of speeding up the test but it should also make the test less sensitive because greater concentrations of antibiotic would be required to arrest the greater number of bacteria.

Anything which might make micro-organisms more sensitive to penicillin would appear therefore merely to increase the sensitivity of the test and not to increase its speed. Had the results with the additions of cobalt and the irradiation of micro-organisms been different to those recorded in this work, this would merely have increased the sensitivity of the test and not resulted in making the test more rapid.

While it is scarcely possible to speed up bacterial growth appreciably, one can speculate on the possibility of observing microbial growth at an earlier stage than in the existing tests. For example, in the disc assay test it might be possible to incorporate a fermentable carbohydrate and a pH indicator in order that early stages in growth could be detected by observing acid formation. If it were desired to affect the intensity of this reaction by increasing the number of micro-organisms, this would have an adverse affect on the sensitivity of the test for antibiotics. Conversely, reducing the number of micro-organisms might render the pH change unobservable. An alternative method might be that of the microscopic examination of plates in order to detect micro-colonies appearing around the zone of inhibition; this might be quite effective with oblique lighting.

At best the resultant saving in time by one or other

of the methods suggested above would not be more than about one hour. This would therefore reduce the length of time of tests from about two and a half hours to about one and a half hours. It is not considered that the time saved would be worthwhile as the nature of tests would not have been altered as the tests would still be completed only after the milk had been delivered and processed at the dairy and could not be used for the rejection of unsatisfactory milk.

The results of the above considerations would seem to point to the fact that it is unlikely that biological tests for antibiotics could be made more rapid. Accordingly, the best policy would seem to be a strong recommandation for the inclusion of dye marking substances in antibiotics used for mastitis so that these can be readily observed on the dairy platform. Such materials have been recommended by several workers including Shahani (1960) and Dawson (1960). This type of screening test coupled with sensitive laboratory tests which would be applied to the product after processing would insure that the screening tests on the dairy platform had been carried out adequately and conscientiously.

REFERENCES

Albright, J.L., Tuckey, S.L. & Woods, G.T. (1961). Antibiotics in milk - A review. J. Dairy Sci., 44, 779.

Arret, B. & Kirshbaum, A.M. (1959). A rapid disc assay method for detecting penicillin in milk. J. Milk Fd Technol., 22, 329.

Berridge, N.J. (1956). Penicillin in milk. II. The incidence of penicillin. J. Dairy Res., 23, 342.

Berridge, N.J. (1956). Rapid routine assay of low concentration of penicillin in milk. J. Dairy Res., 23, 336.

Bruce, C.B. & Mitchell, L. (1952). The effects of surface active agents on bacitracin activity *in vitro*. J. Amer. Pharm. Assoc. (Sci. Ed.), 41, 654.

Burkholder, P.K. & Giles, N.H., Jr. (1947). Induced biochemical mutations in *Bacillus subtilis*. Amer. J. Bot., 34, 345.

Cerny, J. & Morris, R.L. (1955). A modified disc assay method for detecting antibiotics in milk. J. Milk Fd Technol., 18, 281.

Collins, E.B. (1957). A simple method for detecting inhibitory substances in milk. J. Milk Prod., 3, 48.

Cooper, P.D. (1956). Site of action of radiopenicillin. Bact. Rev., 20, 28.

Dawson, D.J. & Feagan, J.T. (1960). The use of Brilliant Blue F.C.F. in intramammary penicillin preparations. Aust. J. Dairy Technol., 15, 160.

Eagle, H. & Saz, A.K. (1955). Antibiotics. Annu. Rev. Microbiol., 9, 173.

Foster, J.W. & Woodruff, H.B. (1943). Microbiological aspects of penicillin. I. Methods of assay. J. Bact., 46, 187.

Galesloot, Th.E. & Hassing, F. (1962). A rapid and sensitive paper disc method for the detection of penicillin in milk. Neth. Milk & Dairy J., 16, 89.

Gogas, W. & Bicknell, A.K. (1953). The determination of penicillin in milk. J. Milk Pl. Mon., 42, 26.

Goldberg, H.S. (1959). Antibiotics Their Chemistry & Non-Medical Uses. D. Van Nostrand Co., Inc., Princeton, N.Y.

Government of Alberta (1960). Ann. Rep.

Grove, D.C. (1959). Prevention of antibiotics in milk - present status. J. Dairy Sci., 42, 199.

Hargrove, R.E., Lehman, R.J. & Matthews, C.A. (1958). Use of fluorescent materials for the indirect detection of antibiotics in milk. J. Dairy Sci., 41, 617.

Hibbs, R.A. & Boyd, J.C. (1957). Some observation on testing milk samples for antibiotics. J. Milk Fd Technol., 20, 109.

Igarashi, R.T., Banghman, R.W., Nelson, F.E. & Hartman, P.A. (1960). Rapid antibiotic assay methods using *Bacillus stearothermophilus*. J. Milk Prod., 9, 16.

Jenness, R. & Patton, S. (1959). Principles of Dairy Chemistry. John Wiley & Sons, Inc., N.Y.

Johns, C.K. (1953). Substances in herd milks inhibiting acid production. Canad. J. Agric. Sci., 33, 586.

Kennedy, H.E. & Harper, W.J. (1960). An approach to a rapid test for antibiotics in milk. J. Dairy Sci., 43, 999.

Kosikowski, F.V. & Mocquot, G.A. (1956). A technique to increase sensitivity of the disc assay method for antibiotics in milk. Proc. Intern. Dairy Congr., Rome, 3, 203.

Kosikowski, F.V. (1957). Controlling growth of test bacteria for antibiotic assay through anaerobiosis. Science, 126, 844.

Kosikowski, F.V. & Ledford, R.A. (1960). A reverse-phase disc assay test for antibiotic in milk. J. Amer. Vet. Med. Assoc., 136, 297.

Kosikowski, F.V. (1963). Induced and natural inhibitory behavior of milk and significance to antibiotic disc assay testing. J. Dairy Sci., 46, 95.

Laboratory Manual (1959). Methods of analysis of milk and its products. Milk Industry Foundation, Washington.

Lederberg, J. & Lederberg, M. (1952). Replica plating and indirect selection of bacterial mutants. J. Bact., 63, 399.

Liska, B.J. (1960). A direct microscopic method for detecting antibiotic in milk. J. Milk Fd Technol., 23, 117.

Marth, E.H. & Ellickson, B.E. (1959). Antibiotic residues in milk and milk products - A review. J. Milk Fd Technol., 22, 241.

Marth, E.H. & Ellickson, B.E. (1959). Problems created by the presence of antibiotics in milk and milk products. J. Milk Fd Technol., 22, 266.

Mei, H.M. & Clegg, L.F.L. (1962). An increase in sensitivity for the assay of penicillin in milk. Nature, 196, 691.

Neal, C.E. & Calbert, H.E. (1955). The use of 2, 3, 5, triphenyltetrazolium chloride as a test for antibiotic substances in milk. J. Dairy Sci., 38, 629.

Neilands, J.B. & Stumpf, P.K. (1955). Outlines of Enzyme Chemistry. John Wiley & Sons, Inc., N.Y.

Pital, A.B.E., Disque, D.T. & Lesise, J.M. (1956). A repid plate method for determining antibiotic sensitivity. Antibiot. & Chemother., 6, 351.

Pratt, R. & Dufrenoy, J. (1948). Cytochemical mechanisms of penicillin action. VI. The influence of cobalt on the optimal bacteriostatic concentration of penicillin. J. Bact., 55, 727.

Prouty, C.C. (1960). A simple and rapid resazurin reduction method with Streptococcus thermophilus for the detection of inhibitory substances in milk. J. Milk Fd Technol., 23, 244.

Pyne, G.T. (1962). Reviews of the progress of dairy science. Section C. Dairy chemistry. Some aspects of the physical chemistry of the salts of milk. J. Dairy Res., 29, 101.

Ruehe, H.A. (1950). Penicillin and other starter inhibitors. J. Milk Pl. Mon., 39, 36.

Schipper, A.I. & Peterson, W.E. (1951). Assay of aureomycin in milk by use of methylene blue. Vet. med. Assoc., 46, 222.

Shahani, K.M. (1958-1959). Visual detection of antibiotics in milk by means of dye. Antibiotics Ann. p.883. Medical Encyclopedia, Inc., N.Y.

Shahani, K.M. (1960). Proc. Milk Industry Foundation Convention, Laboratory Section, Chicago.

Shahani, K.M. & Badami, M.C. (1958). A resazurin disc assay method for detecting antibiotic and natural starter inhibitory activity in milk. J. Dairy Sci., 41, 1510.

Silverman, G.J. & Kosikowski, F.V. (1952). Systematic testing of inhibitory substances in milk. J. Milk Fd Technol., 15, 120.

Smitasiri, T., Kosikowski, F.V., Guthrie, R.S. & Fincher, M.G. (1958). Dyes as markers for antibiotic contaminated milks. J. Milk Fd Technol., 21, 255.

Standard Methods for the Examination of Dairy Products, 10th Ed. 1953, American Public Health Assoc., N.Y.

Storgards, T. & Mohr, W. (1962). Detection of penicillin and other antibiotics in milk. International Dairy Federation, Ann. Bull., part III, 4.

Storrs, F.C. & Hiett-Brown, W. (1954). The incidence of penicillin in milk supplies. J. Dairy Res., 21, 337.

Thatcher, F.S. (1962). Personal communication.

Trembath, R.H. (1950). Effect of penicillin in milk on starter cultures. Aust. J. Dairy Technol., 5, 143.

Welch, H. (1957). Problems of antibiotics in food as the food and drug administration sees them. Amer. J. public Hlth., 47, 701.

Whitehead, H.R. & Cox, G.A. (1956). The detection of penicillin in milk. J. appl. Bact., 19, 247.